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Reuse of polluting agroindustrial waste for ethanol production by *Kluyveromyces marxianus*

Joselma Ferreira da Silva[a], Naiara Priscila Silva Reis Barbosa[a], Matheus Tavares do Nascimento França[b], Laureen Michelle Houllou[a], Carolina Barbosa Malafaia[a]

[a] Centro de Tecnologias Estratégicas do Nordeste-CETENE, Av. Prof. Luís Freire, 01, Cidade Universitária, Recife, PE, Brasil. CEP: 50.740-540. Corresponding author: joselma.fsilva@gmail.com.

[b] Instituto Federal de Pernambuco-IFPE, Av. Prof. Luís Freire, 500, Cidade Universitária, Recife, PE, Brasil. CEP 50.740-540.

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ABSTRACT

The development of research for the production of biofuels using low-cost substrate has become more relevant in recent years. These include reuse of residues such as crude residual glycerol from biodiesel (CRG) and cheese whey (CW) from the dairy industry. This study evaluated the ethanol production by isolates of the yeast *Kluyveromyces marxianus* using agroindustrial residues as an alternative source of carbon. The cultures were rotated 100 rpm at 30°C for 24 h. The ethanol production was observed in both media; however, in the CW higher values of ethanol was observed about the CRG. The results showed that *K. marxianus* isolates were adapted to the use of lactose present in cheese whey as a source of carbon for the production of ethanol with concentrations ranging from 11.41 to 19.9 $g.L^{-1}$, but did not demonstrate efficiency in the use of crude glycerol for this purpose.

Keywords: Fermentation, cheese whey, glycerol, biofuel, yeast.

Introduction

The progressive increase in the use of fossil fuels, which is currently the most widely used energy source in the world, has encouraged the increase in the search for new sources of renewable, sustainable, economically and environmentally favorable sources of energy (Soccol et al., 2009). The production of biofuels by biological conversion has been growing as a promising strategy, seeking to meet the demand demanded by society for presenting positive factors such as the reduction of emissions of pollutants and higher economic stability (Banerjee et al., 2010; Behera et al., 2014).

Bioethanol is a fuel considered as a promising alternative to clean energy because of its high conversion energy (Arora et al., 2015; Kumar et al., 2010). The interest of researchers in the use of bioethanol as an energy source has stimulated studies on the cost and efficiency of industrial processes for their production. Currently, the challenge is to use cheaper carbon sources as a substrate for their production. In this sense, the use of organic pollutants can be a viable alternative for this purpose (Behera et al., 2016).

The activities carried out by the industrial processes lead to the generation of potentially polluting residues during their production, which is usually not correctly treated (Meneses, 2009), causing severe environmental problems, mainly because of the present, in general, a high concentration of organic matter. Among the by-products generated, there are dairy residues, Cheese Whey (CW), which is one of the most important, due to the generated volume and high biochemical oxygen demand ranging from 30 to 50 $g.L^{-1}$ (Prazeres et al., 2012). Another critical highly polluting residue is the crude residual glycerol (CRG) from the production of biodiesel (Yang et al., 2012). The bioconversion of these residues by biotechnological processes generates higher added value products, such as biomass and biomolecules, being a relevant alternative for the reuse of this waste.

Saccharomyces cerevisiae is one of the most widely used microorganisms in the bioethanol production industry (Mohd Azhar et al., 2017), but this microorganism is not able to metabolize the lactose present in cheese whey, due to the absence of the enzyme that degrades lactose is a molecular mechanism of repression on other enzymatic

pathways for assimilation of other sugars (Guimarães et al., 2010). Searching for alternative microorganisms to produce ethanol using residues, several authors have proposed that *Kluyveromyces marxianus* yeasts, due to their potential to produce ethanol using cheese whey as a carbon source, considered as a potential for the bioconversion of lactose in ethanol (Zoppellari & Bardi, 2012).

Kluyveromyces spp. are fermentative-fermenting yeasts capable of generating energy through respiration or fermentation (Abdel-Banat et al., 2009; Guimarães et al., 2010). These offer benefits such as high growth rate, rapid cell multiplication, safe use, ability to use industrially relevant substrates such as sugar cane, molasses, cheese whey, corn, and glycerol (Abdel-Banat et al., 2009; Guimarães et al., 2010; Kádár et al., 2011; Koushki et al., 2012).

Therefore, the objective of this study was to evaluate the ethanol production by isolates of *K. marxianus* in fermentations using agroindustrial residues as alternative sources of carbon.

Material and Methods

Microorganism

Isolates of *Kluyveromyces marxianus* (CTN-30, CTN-32, CTN-113, CTN-374, CTN-412) obtained by direct isolation of dairy from the Pernambuco region were used. Yeasts were isolated by depletion in Petri dishes containing YPDA medium (yeast extract - 10 $g.L^{-1}$, peptone - 20 $g.L^{-1}$, dextrose - 20 $g.L^{-1}$, Agar - 20 $g.L^{-1}$) with the antibiotic, were identified through the protein profile using the Matrix Associated Laser Desorption-Ionization Time of Flight technique (MALDI-TOF-Bruker). After isolation, the strains were maintained in YPD medium at 30°C at 150 rpm for 24 h.

Preparation of the inoculum

The starting inoculums were prepared according to the residue to be used in the fermentation. They were prepared in two media in 250 mL Erlenmeyer flasks, containing 100 mL in YSG medium (yeast extract - 10 $g.L^{-1}$, ammonium sulfate - 5 $g.L^{-1}$, crude residual glycerol - 20 $g.L^{-1}$) or previously sterilized cheese without added nutrients. The inoculated vials were incubated in the shaker at 30°C and 150 rpm for 24 h. The inoculum constituted 10% of the final volume of the culture standardized by optical density (OD) of 0.8 to 0.9 to 600nm.

Fermentation

The fermentation was carried out using the same means previously mentioned and incubated under the same conditions. To evaluate microbial growth, pH, and ethanol production samples of 3 mL were collected every 3 h from time 0 to 24 h. 2.4.

Growth kinetics

Growth curves of the isolates were determined in 250 mL Erlenmeyer cells, with a volume of 125 mL, using a cell inoculum of 0.5 (OD600 nm), for 24 h at 30°C, and shaking at 150 rpm. The 1 mL aliquots were collected and centrifuged at 5000 x g for 15 min; the cells were washed with sterile distilled water and resuspended in 1 ml of 0.9% saline. The cell concentration was determined by optical density (OD 600nm).

Determination and pH

The pH was determined using a model digital potentiometer (pH 1800 PG, Gehaka), at room temperature 25°C using 3 mL of the cell-free fermented broth, centrifuged at 5000 x g for 10 min.

Determination of ethanol

Determination of the ethanol content was performed on a gas chromatograph (Agilent Technologies model 7890A) equipped with a flame ionization detector and coupled to a DB-WAX capillary column (30 m x 0.32 mm x 0.25 μm Agilent Technologies). The injection volume was 1 μL, with a division rate of 100:1. The oven temperature was kept constant at 40°C for 6 min, and the injection and detection temperatures at 250°C. The quantification was performed from the calibration curve.

Results

Figure 1 shows the growth curves and growth just after 3 h and with maximum growth peak of all the isolates, except CTN-30, that occurred at 21 h in both media tested. CTN-32 showed the highest cell density in CW and CTN-374 in YSG medium at the same culture time.

The isolates had better performance in the CW medium when compared to the YSG. It can then be said that the yeast *K. marxianus* toabout the cheese whey was efficient in its cellular production having as sole carbon source the chesse whey nutrients of the cheese. After this period in both media, the concentration of biomass decreases, signaling that the yeasts have begun the phase of death or decline.

The pH in both media tested showed that the isolates, during the growing period, showed a similar profile, is possible to observe their tendency to reduce pH in the period of 24 h of culture (Figure 2). There was variation in CW from 3 h of culture, already in the YSG only from 15 h.

As for ethanol production (Figure 3), it was observed that after 24 h of fermentation, the highest production occurred with the CTN-30 isolate, reaching satisfactory results with the production of 19.9 g.L^{-1} ethanol in the residue CW. The YSG did not show any significant variations. The yeasts presented an unsatisfactory correlation between glycerol and ethanol production, as we can see in Figure 3b.

Figure 1. Growth kinetics in CW (A) and YSG (B) culture media. -■-CTN-30, -●-CTN-32,-▲CTN-113, -▼-CTN-374, -◀-CTN-412.

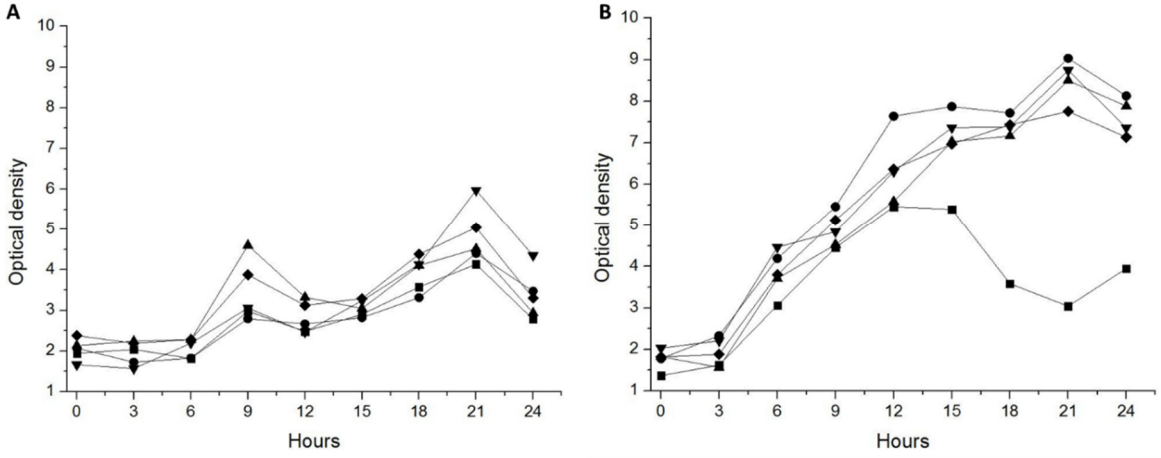


Figure 2. pH variation over the growing period CW (A) and YSG (B). -■-CTN-30, -●-CTN-32,-▲CTN-113, -▼-CTN-374, -◀-CTN-412.

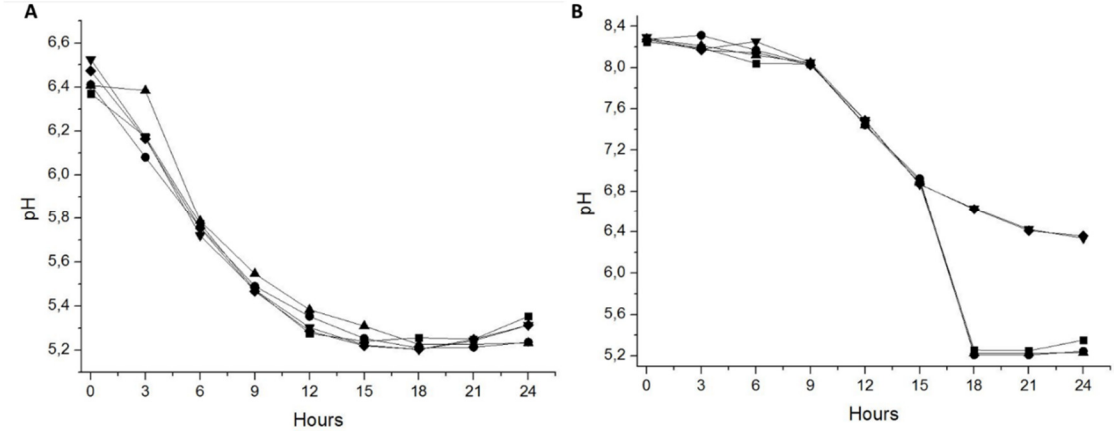
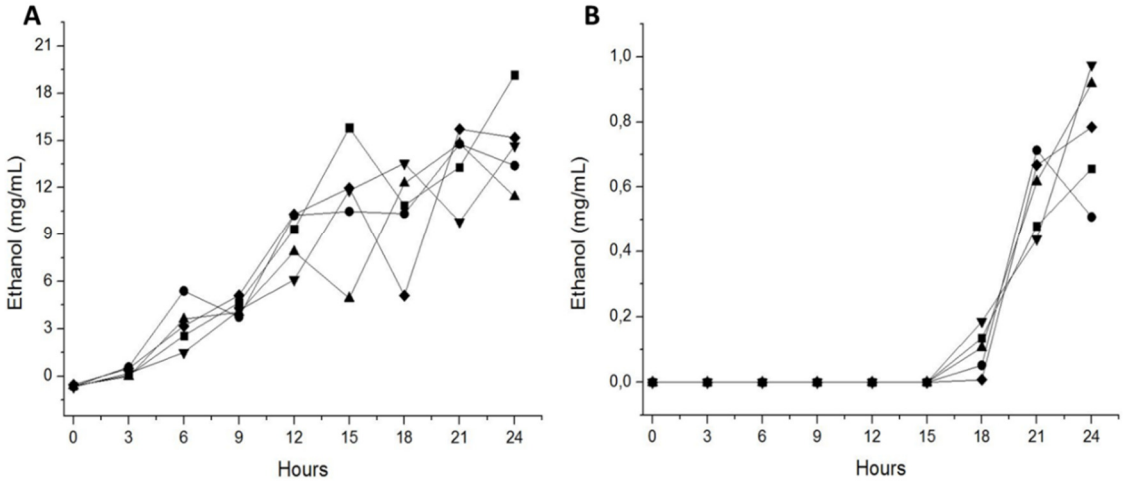


Figure 3. Ethanol production over the growing period CW (A) and YSG (B). -■-CTN-30, -●-CTN-32,-▲-CTN-113, -▼-CTN-374, -◀-CTN-412.



Discussion

The cheese whey was used in two agroindustrial residues, and a lactose-rich substrate is a fermentable sugar that can be used as a source of carbon in the fermentation process. Glycerol is a substrate considered as a precursor of several compounds and regulator of various intracellular mechanisms and assimilated by active transport in the cell (Siqueira, 2015).

The fermentative metabolism of the yeast *K. marxianus* can channel all the sugar by fermentation and plays a fundamental role in the production of ethanol using the cheese whey and crude glycerol. In the present study, it is possible to obtain lactose as a source of carbon (Abdel-Banat et al., 2009), making the use of cheese whey efficient for the production of ethanol, thus becoming one of the possible solutions for cheese bioremediation (Saini et al., 2017).

The cell growth of *K. marxianus* can be justified by the fact that these microorganisms were isolated from dairy farming in Pernambuco, so the isolates tested were well adapted to the use of lactose as a source of carbon. It is known that few yeasts are able to metabolize the lactose present in cheese whey in ethanol, but due to the presence of LAC12 and LAC4 genes in *K. marxianus*, which are responsible for the expression of lactose-permease enzymes, which act in the transport of lactose through the plasma membrane into the cell (Guimarães et al., 2010).

The reduction of pH occurs due to the production of ethanol and the formation of organic acids that consequently acidify the medium. It is an expected result of the fermentation process due to the metabolization of sugars that cause its release into the cytoplasm, influencing the consumption of sugars and the bioconversion of lactose in ethanol (Telli Okur & Eken Saraçoğlu, 2006). In previous studies, such as that carried out by Bitello et al. (2013), there was also a reduction in pH from 6.5 to 4.5 in 48 h of fermentation.

Notably, the ethanol production obtained when CW fermentations presented similar results to those obtained by Sansonetti et al. (2010), that after 15 h of fermentation produced 22.68 $g.L^{-1}$ of ethanol. Murari et al. (2017) state that *K. marxianus* obtained satisfactory yield with values above 20 $g.L^{-1}$ and good performance in the fermentation of the cheese whey. The values found are within the expected range since the literature describes similar values. In the studies carried out by Ozmihci & Kargi (2007), cheese whey concentrate (12.5% lactose) was used as the substrate for the production of ethanol from *K. marxianus* of a batch fed, reaching the productivity of 5.3 $gL^{-1}.h^{-1}$ of ethanol at 6.3% (m/v).

In this way, the production of bioethanol with *Kluyveromyces marxianus* using cheese whey guaranteed the necessary nutrients for growth and fermentation due to the rich lactose medium. It is known that sugar influences the fermentation of this yeast (Diniz et al., 2017).

Conclusion

The bioconversion capacity of the agroindustrial residues for ethanol production is evidenced using *Kluyveromyces marxianus*, and it is promising in the energy requirements. The use of cheese whey as a source of carbon proved to be more efficient than crude residual glycerol. It can become a consistent alternative for the production of economically competitive ethanol, besides having as an associated advantage the reuse of pollutants, avoiding environmental contamination.

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